



## Impact of external carbon dose on the removal of micropollutants using methanol and ethanol in post-denitrifying Moving Bed Biofilm Reactors

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19 **Abstract**

20 Addition of external carbon sources to post-denitrification systems is frequently used in wastewater  
21 treatment plants to enhance nitrate removal. However, little is known about the fate of  
22 micropollutants in post-denitrification systems and the influence of external carbon dosing on their  
23 removal. In this study, we assessed the effects of two different types and availability of commonly  
24 used carbon sources —methanol and ethanol— on the removal of micropollutants. Two laboratory-  
25 scale moving bed biofilm reactors (MBBRs), containing AnoxKaldnes K1 carriers with acclimated  
26 biofilm from full-scale systems, were operated in continuous-flow using wastewater dosed with  
27 methanol and ethanol. Batch experiments with 22 spiked pharmaceuticals were performed to assess  
28 removal kinetics. Acetyl-sulfadiazine, atenolol, citalopram, propranolol and trimethoprim were  
29 easily biotransformed in both MBBRs (biotransformations rate constants  $k_{\text{bio}}$  between 1.2 and 12.9  
30  $\text{L g}_{\text{biomass}}^{-1} \text{d}^{-1}$ ), 13 compounds were moderately biotransformed (rate constants between 0.2 and 2  $\text{L}$   
31  $\text{g}_{\text{biomass}}^{-1} \text{d}^{-1}$ ) and 4 compounds were recalcitrant. The methanol-dosed MBBR showed higher  $k_{\text{bio}}$   
32 (e.g., 1.5 to 2.5-fold) than in the ethanol-dosed MBBR for 9 out of the 22 studied compounds, equal  
33  $k_{\text{bio}}$  for 10 compounds, while 3 compounds (i.e., targeted sulfonamides) were biotransformed faster  
34 in the ethanol-dosed MBBR. While biotransformation of most of the targeted compounds followed  
35 first-order kinetics, removal of venlafaxine, carbamazepine, sulfamethoxazole and sulfamethizole  
36 could be described with a cometabolic model. Analyses of the microbial composition in the  
37 biofilms using 16S rRNA amplicon sequencing revealed that the methanol-dosed MBBR contained  
38 higher microbial richness than the one dosed with ethanol, suggesting that improved  
39 biotransformation of targeted compounds could be associated with higher microbial richness.  
40 During continuous-flow operation, at conditions representative of full-scale denitrification systems  
41 (hydraulic residence time = 2 h), the removal efficiencies of micropollutants were below 35% in  
42 both MBBRs, with the exception of atenolol and trimethoprim (>80%). Overall, this study

43 demonstrated that MBBRs used for post-denitrification could be optimized to enhance the  
44 biotransformation of a number of micropollutants by accounting for optimal carbon sources and  
45 extended residence time.

46

## 47 **1. Introduction**

48 Currently used biological processes in conventional wastewater treatment plants (WWTPs) are  
49 designed to remove organic carbon and nutrients (nitrogen and phosphorus). As organic  
50 micropollutants are gaining attention due to the associated environmental risks (Daughton and  
51 Ternes, 1999; Plósz et al., 2013), the optimization of biological processes for removal of  
52 micropollutants during wastewater treatment is crucial (Joss et al., 2008). Micropollutants (e.g.,  
53 pharmaceuticals and personal care products) are generally recognized as non-growth substrates  
54 (secondary substrates), as they are present in wastewater in too low concentrations ( $\text{ng L}^{-1}$  to  $\mu\text{g L}^{-1}$ )  
55 to support biomass growth (Fischer and Majewsky, 2014; Rittmann, 1992). Therefore, biological  
56 transformation of micropollutants is mainly the result of cometabolic mechanisms, whereby the  
57 removal of non-growth substrates (micropollutants) requires the presence of primary substrates (i.e.,  
58 COD, nutrients) to support biomass growth (Criddle, 1993; Rittmann, 1992). In cometabolism, the  
59 biotransformation of micropollutant is typically catalyzed by non-specific enzymes (e.g., mono- or  
60 di-oxygenases, *N*-acetyltransferases, hydrolases) or by cofactors produced during the microbial  
61 conversion of the primary substrate (Criddle, 1993; Fischer and Majewsky, 2014). Nevertheless, the  
62 interaction between primary substrate and micropollutants is complex and not completely  
63 understood. In fact, the presence of primary substrate has been reported to either enhance the  
64 removal of micropollutants, e.g., by regenerating reductants such as NAD(P)H under aerobic  
65 conditions (Alvarez-Cohen and Speitel, 2001; Liu et al., 2015), or decrease it, due to competitive  
66 enzyme inhibition (Fischer and Majewsky, 2014; Plósz et al., 2010).

67

68 Recent studies have proposed biofilm systems, e.g., moving bed biofilm reactors (MBBR), as a  
69 promising alternative to activated sludge systems (CAS) with respect to the attenuation of  
70 micropollutants (Escolà Casas et al., 2015a; Falås et al., 2012; Hapeshi et al., 2013; Torresi et al.,

71 2016). In general, most of the studies concerning the removal of micropollutants during biological  
72 wastewater treatment have focused on aerobic systems, whereas only little information is available  
73 for anoxic denitrifying conditions: Plósz et al., 2010; Su et al., 2015; Falås et al. 2013, Suárez et al.,  
74 2010. While pharmaceuticals such as diclofenac, metoprolol, erythromycin and roxithromycin were  
75 found to be transformed mainly under aerobic conditions in activated sludge using synthetic  
76 wastewater (Suárez et al., 2010) and in hybrid biofilm-activated sludge processes (Falås et al.,  
77 2013), some of the investigated chemicals had similar (i.e., bezafibrate, atenolol, clarithromycin and  
78 N<sup>4</sup>-acetylsulfamethoxazole) or higher (i.e., levetiracetam) biotransformation under anoxic  
79 conditions than under aerobic ones (Falås et al., 2013). Hence, anoxic biological processes in  
80 conventional WWTPs should be considered as a potential step to optimize removal of  
81 micropollutants.

82 The type of carbon source is known to have a strong impact on the structure of denitrifying  
83 microbial communities and thus on denitrification efficiency (Baytshok et al., 2009; Hagman et al.,  
84 2007; Lu et al., 2014). This has specific relevance to post-denitrification reactors in full-scale  
85 WWTPs, where nitrate removal is achieved by dosing external carbon sources such as methanol and  
86 ethanol (Louzeiro et al, 2002; Santos et al., 2001). Methanol and ethanol are metabolized by  
87 denitrifying bacteria through different pathways. Methanol undergoes the metabolic reaction of  
88 single-carbon compounds, which is exclusive to methylotrophs because of their unique key enzyme  
89 (methanol dehydrogenase) that catalyzes the oxidization of methanol to formaldehyde (Anthony et  
90 al., 1982, 2011). Instead, ethanol is easily converted by bacterial cells to Acetyl-CoA before  
91 entering the glyoxylate cycle (Anthony et al., 2011). Thus, microbial communities in post-  
92 denitrifying systems using either of these carbon sources may be fundamentally different and  
93 potentially exhibit a different biodiversity and functionality with more or less microbial specialists  
94 able to biotransform organic micropollutants. Additionally, biodiversity in terms of species richness

95 (the number of species) and evenness (the relative abundance of the species) (Wittebolle et al.,  
96 2009) was shown to positively associate with the biotransformation of a number of micropollutants  
97 in aerobic activated sludge (Johnson et al., 2015; Stadler and Love, 2016) and nitrifying MBBRs  
98 (Torresi et al., 2016). Further investigation of the impact of biodiversity in different biological  
99 treatment systems seems thus required.

100 In this study, we evaluated the elimination of selected micropollutants (i.e., pharmaceuticals) in  
101 laboratory-scale post-denitrifying MBBRs dosed with methanol or ethanol. Biotransformation  
102 kinetics and removal efficiencies were assessed through targeted batch experiments and during  
103 continuous-flow MBBR operation, respectively. The objectives of our study were: (i) to investigate  
104 the impact of different types of external carbon sources (methanol and ethanol) for post-  
105 denitrification on micropollutant biotransformation; (ii) to assess the structure of the denitrifying  
106 microbial community of MBBR biofilms, following continuous dosing with either methanol or  
107 ethanol; and (iii) to evaluate the influence of organic substrate availability on the transformation of  
108 micropollutants and the related mechanisms, i.e. competitive inhibition and cometabolic  
109 enhancement.

110

## 111 **2. Materials and Methods**

112

### 113 **2.1 Description of the post-denitrifying systems**

114 Two Swedish WWTPs, i.e., Sjölanda and Klagshamn are currently dosing methanol or ethanol,  
115 respectively, as external carbon source in two-stage post-denitrification MBBRs. Thus, two  
116 laboratory-MBBRs were built to resemble such post-denitrification stages, using carriers  
117 (AnoxKaldnes K1) from the first post-denitrification tank of the respective WWTPs already adapted  
118 to methanol and ethanol dosing. The WWTPs are described in Section S1 of the SI (Supporting

119 Information). Both laboratory-MBBRs (1 L) were operated in continuous feeding of the same  
120 wastewater, which was collected after the (aerobic) nitrification step (trickling filter) of Sjölanda  
121 WWTP (Lund, Sweden). Thus, during continuous-flow operation, only the indigenous nitrate and  
122 nitrite present in the wastewater (averaged concentration of 13 and 1.2 mg L<sup>-1</sup> respectively) were  
123 used for denitrification. The filling rate of both reactors was 40%, giving a surface of 0.2 m<sup>2</sup>. The  
124 amount of indigenous micropollutants in the reactor influents (consisting in the collected  
125 wastewater and feed containing carbon-source) was analyzed. Twelve compounds were quantified  
126 giving concentrations between 0.04 µg L<sup>-1</sup> (trimethoprim) and 78 µg L<sup>-1</sup> (iohexol). Complete details  
127 of these results are given in Table S6 (SI).

128

129 The reactors were continuously flushed with nitrogen gas and stirred for the mixing of the carriers  
130 and to strip eventual residual dissolved oxygen. Both reactors were kept at 15°C using a water bath.  
131 The feed wastewater was mixed and kept at 4°C during the whole experiment. Phosphate was added  
132 to the feed to reach a concentration of 0.5 mg L<sup>-1</sup> to ensure biofilm growth on the carriers.  
133 Micropollutant removal and denitrification rates in MBBRs were assessed in two main experiments:  
134 (i) batch conditions (24 h) and (ii) continuous-flow operation (2 months). The carbon availability of  
135 ethanol and methanol into the two MBBRs was defined as the ratio between the influent loading of  
136 organic carbon (COD<sub>added</sub>) and the native loading of nitrate (NO<sub>3</sub>-N<sub>influent</sub>) in the wastewater  
137 samples. Optimum COD<sub>added</sub>/NO<sub>3</sub>-N<sub>influent</sub> ratio (gCOD gN<sup>-1</sup>) for complete denitrification is typically  
138 around 4 (Metcalf & Eddy, 2003).

139

## 140 **2.2 Analytical methods**

141 All the samples taken for analysis of conventional pollutants (NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, NO<sub>2</sub><sup>-</sup>-N, soluble  
142 COD and PO<sub>3</sub><sup>4-</sup>) in batch and continuous experiments were filtered through 0.45 µm glass fiber

143 filters (Sartorius, Göttingen, Germany). Total COD and total nitrogen were analyzed on the  
144 unfiltered sample. All samples were prepared in Hach Lange kits (LCK 303, LCK 339, LCK 341  
145 and LCK 342) and analyzed in a Hach Lange DR2800 spectrophotometer. DO, pH and temperature  
146 in the reactors were measured at each sampling occasion, using a Hach HQ40d multi DO probe and  
147 a HANNA H1991001 pH-meter. The attached biomass concentrations were calculated from the  
148 difference in weight of 3 dried carriers (105 °C for >24 h) before and after biofilm removal (in 2M  
149 H<sub>2</sub>SO<sub>4</sub>) with subsequent brushing (see Figure S2 for results), as previously considered (Escolà  
150 Casas et al., 2015a; Falås et al., 2012; Torresi et al., 2016). Samples for micropollutants were frozen  
151 at -20 °C prior analysis and analyzed via direct injection using HPLC-MS/MS as described in  
152 Escolà Casas et al. (2015a). Information regarding sample preparation, HPLC, mass spectrometry  
153 data, LOD and LOQ of compounds are shown in Escolà Casas et al. (2015a) and in Section S2 (SI).

154

### 155 **2.3 Chemicals**

156 Twenty-two relevant micropollutants (i.e., pharmaceuticals) were selected for this study.  
157 Information regarding CAS numbers and chemical suppliers is found in the supplementary  
158 information in Escolà Casas et al. (2015b). The pharmaceuticals included: (i) four beta-blockers,  
159 i.e., atenolol, metoprolol, propranolol and sotalol; (ii) five X-ray contrast media, i.e., diatrizoic acid,  
160 iohexol, iopamidol, iopromide, iomeprol; (iii) three sulfonamides, i.e., sulfadiazine, sulfamethizole  
161 and sulfamethoxazole and the metabolite acetyl-sulfadiazine; (iv) three analgesics, i.e., phenazone,  
162 diclofenac and ibuprofen; (v) three anti-epileptics/anti-depressants, i.e., carbamazepine, venlafaxine  
163 and citalopram; (vi) four antibiotics, i.e., erythromycin, clarithromycin, trimethoprim and  
164 roxithromycin.

165

166

## 167 **2.4 Batch experiment**

168 To investigate how the type of dosed carbon source influences the removal of micropollutants in  
169 post-denitrifying MBBR, batch experiments were performed in the same reactors used during  
170 continuous-flow operation. These experiments were conducted after 3.5 months of continuous-flow  
171 operation of the two systems. During the batch experiments, a  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratio of 3.4 for  
172 both reactors was adopted to obtain excess concentration of nitrate. Anoxic conditions were  
173 maintained by flushing the reactors with nitrogen gas during the experiment. The feed used for the  
174 batch consisted of the same wastewater used in continuous operation spiked with  $239 \pm 2$  mg  
175  $\text{COD L}^{-1}$  of methanol for the methanol-dosed reactor and the same amount of ethanol for the  
176 ethanol-dosed reactor,  $70 \pm 3$  mg  $\text{NO}_3\text{-N L}^{-1}$  in form of sodium nitrate and 22 micropollutants with  
177 an initial nominal concentration of  $2 \mu\text{g L}^{-1}$ . The micropollutants were added from a stock solution  
178 ( $40 \text{ mg L}^{-1}$  in methanol). To minimize the increase of COD concentration in the batch feed due to  
179 the methanol from the stock solution, the micropollutant solution was first spiked into an empty  
180 glass beaker and the methanol was let to evaporate for approximately 1 hour. Afterwards, the feed  
181 was added to the beaker containing the micropollutants and mixed to re-dissolve the  
182 micropollutants. The batch experiment lasted 24 hours and samples for conventional and  
183 micropollutants analysis were taken at regular intervals. To keep the biomass concentration constant  
184 during the experiment, three carriers were withdrawn from the reactors each time a sample was  
185 taken for analysis. The pH value in both reactors was continuously measured and adjusted to 7.5  
186 using 1M HCl. The temperature was kept constant at  $15^\circ\text{C}$ .

187

### 188 ***2.4.1 Denitrification during batch experiment***

189 Denitrification rates normalized on surface area of reactors  $r_{\text{NO}_3,2\text{-N}}$  ( $\text{gNO}_{3,2\text{-N}} \text{ m}^{-2} \text{ d}^{-1}$ ) and specific  
190 denitrification rates accounting for the biomass  $k_{\text{NO}_3,2\text{-N}}$  ( $\text{gNO}_{3,2\text{-N}} \text{ g}_{\text{biomass}}^{-1} \text{ d}^{-1}$ ) were derived

191 through linear regression using  $\text{NO}_3^-$ -N and  $\text{NO}_2^-$ -N measurements during batch experiment. An  
192 accumulation of nitrite in the systems was noticed ( $\sim 6 \text{ mg L}^{-1}$ ), therefore  $\text{NO}_{3,2}$ -N utilization curves  
193 accounting also for  $\text{NO}_2^-$ -N concentration were derived accordingly to Sözen et al. (1998).

194

195 A two step-denitrification activated sludge model (ASM) was used to describe up-take of primary  
196 substrates (i.e., readily soluble biodegradable COD ( $S_S$ ), soluble nitrate ( $S_{\text{NO}_3}$ ) and nitrite ( $S_{\text{NO}_2}$ ))  
197 which was extended with the Activated Sludge Model for Xenobiotics ASM-X (Plósz et al., 2012;  
198 Polesel et al., 2016) to determine micropollutant biotransformation rates (Table 1). Readily soluble  
199 biodegradable COD ( $S_S$ ) was determined as the difference between soluble COD (sCOD)—  
200 measured during the experiments—and soluble inert COD ( $S_I$ )—calculated according to Roeleveld  
201 and Van Loosdrecht (2002). The ASM for denitrification was adapted from Pan et al. (2015) and  
202 included two process rate equations with reduction of nitrate to nitrite (R1) and nitrite to nitrogen  
203 (R2) (Table 1). Parameters that could not be identified through model calibration to experimental  
204 results (maximum specific growth rates  $\mu_H$ , affinity constants for substrate— $K_{S1}$  and  $K_{S2}$ —and for  
205 nitrogen species— $K_{\text{NO}_3}^{\text{HB}}$  and  $K_{\text{NO}_2}^{\text{HB}}$  —) were adopted from literature (Hiatt and Grady, 2008).  
206 Parameters that are known to be sensitive to the experimental data (i.e., heterotrophic yields  $Y_H$ ,  
207 anoxic growth factors for the process 1 and 2,  $\eta_{g1}$  and  $\eta_{g2}$ ) were calibrated. Definition of the  
208 components and model calibration are presented in Section S3 and Table S1 (SI). The model was  
209 implemented in AQUASIM 2.1d (Reichert et al., 1994) and the parameters were estimated using the  
210 secant method embedded.

211

#### 212 ***2.4.2 Micropollutants removal kinetics during batch experiment***

213 Model structures to assess biotransformation rate of micropollutants were identified using the ASM-  
214 X as modelling framework (Polesel et al., 2016; Plósz et al., 2010, 2012, 2013). The framework

215 used in this study is summarized in Table 1 and included processes such as parent compound  
216 retransformation (e.g., deconjugation of human metabolites) (1), biotransformation (2) and  
217 cometabolic biotransformation (in the presence and absence of organic growth substrate) (3). The  
218 effect of diffusion into biofilm on the removal of pharmaceuticals from bulk aqueous phase was  
219 lumped in the biotransformation rate constants, as previously considered by Falås et al. (2012,  
220 2013), Escolá Casas et al. (2015a) and Hapeshi et al. (2013). The cometabolic process was  
221 modelled as proposed by Plósz et al. (2012), using pseudo-first order kinetics with respect to  
222 micropollutant concentration and estimating two biokinetics: (i) the cometabolic biotransformation  
223 rate constant  $q_{\text{bio}}$  in the presence of the primary substrate and (ii) biotransformation rate constant  
224  $k_{\text{bio}}$  in the absence of primary substrate. Accordingly, biotransformation kinetics of the cometabolic  
225 substrate (e.g., micropollutant) depend on the primary substrate concentration (e.g., organic matter  
226 expressed as readily soluble biodegradable COD,  $S_S$ ) considered a co-limiting substrate. In Table 1  
227  $C_{\text{LI}}$  and  $C_{\text{CJ}}$  denote the aqueous concentration ( $\text{ng L}^{-1}$ ) of the parent compound and the human  
228 metabolites undergoing deconjugation to the parent compound, respectively. The retransformation  
229 rate constant  $k_{\text{Dec}}$  ( $\text{L g}_{\text{biomass}}^{-1} \text{d}^{-1}$ ) defines kinetics of retransformation to parent compound. Sorption  
230 processes were also included considering the sorption coefficient  $K_D$  ( $\text{L g}_{\text{biomass}}^{-1}$ ) which was  
231 calibrated using values from previous studies estimated under denitrifying condition when available  
232 (Table S2 in SI). As to the best of our knowledge values of  $K_D$  were not previously estimated for  
233 biofilm under denitrifying conditions,  $K_D$  measured for activated sludge were used in this study.  
234 The half-saturation coefficient for  $S_S$ , ( $K_S$ ) in Table 1, was retrieved from Hiatt and Grady (2008).  
235  $X_{\text{biomass}}$  ( $\text{g}_{\text{biomass}} \text{L}^{-1}$ ) denoted the biomass concentration in the MBBRs and growth of biomass on  
236 micropollutants was considered negligible.

237

238 Biotransformation constants  $k_{\text{bio}}$  (process 2, Table 1) were estimated from the measured data using  
239 least-square optimization without weighting in GraphPad Prism 5.0. Parent compound  
240 retransformation and cometabolism model (processes 1 and 3, Table 1) were implemented in  
241 AQUASIM 2.1d (Reichert et al., 1994) and the parameters were estimated using the secant method  
242 embedded.

243 Removal rate constants  $k$  ( $\text{d}^{-1}$ ) were also estimated to compare the performance of the two MBBR  
244 systems, regardless of biomass concentration and sorption processes (Escolà Casas et al., 2015a,b).  
245 For the chemicals following cometabolism model (and thus exhibiting two biokinetics),  $k$  was  
246 calculated considering the estimated  $q_{\text{bio}}$ . Differences between biotransformation rate constants of  
247 the two MBBRs were assessed by examining the overlap between standard deviations of the  
248 estimated values (Cumming et al., 2007).

249

## 250 **2.5 DNA extraction, PCR amplification, sequencing and bioinformatics analysis.**

251 One carrier was collected from each MBBR before the batch experiment and stored in a sterilized  
252 Eppendorf tube at  $-20$  °C. Biomass was detached using a sterile brush (Gynobrush, Dutscher  
253 Scientific, United Kingdom) using tap water and consequently centrifuged (10000 rpm for 5  
254 minutes) to remove excess water. DNA extraction, PCR amplification (using 16S rRNA bacteria  
255 gene primers) and Illumina sequencing were performed as described in Section S5 of the SI.  
256 Taxonomic assignment and calculation of alpha diversity metrics (Shannon biodiversity and ACE  
257 extrapolated richness) were performed in mothur using the RDP reference taxonomy. Additional  
258 diversity indices were calculated according to Hill et al. (1973). Microbial evenness was estimated  
259 as  $H_1/H_0$  as described in Johnson et al. (2015).

260

## 261 2.6 Continuous-flow experiment

262 The two MBBRs used for the present study were operated for over 4 months. The MBBRs were  
263 kept with a  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratio equal to 3 (close to the ratios used at the respective  
264 WWTPs) for the first two weeks of operation. The fraction of inert COD was taken into account (by  
265 subtracting it from the amount of available biodegradable COD) when planning experiments under  
266 carbon limitation. A hydraulic retention time (HRT) of 2 hours was set similar to the HRT used at  
267 the full-scale WWTPs. After two weeks of acclimatization, baseline carbon-dosage periods of  
268  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  were alternated with short carbon-dosage periods (~5 days) to avoid biomass  
269 adaptation. Accordingly, concentrations of methanol and ethanol in the feed solutions were changed  
270 to test  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratios ranging from 1 to 5, while keeping constant HRT. This test  
271 phase lasted about 2 months. The  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratios and the dates are reported in the  
272 Table S3 (SI). The range of  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratios was chosen to assess carbon limiting  
273 condition at low  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratio and not far exceeding the stoichiometric  
274  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratio needed for complete denitrification. The denitrification rate  $r_D$  ( $\text{gN d}^{-1}$   
275  $\text{m}^{-2}$ ) in continuous operation, was calculated for each carbon-dosage test by using the Equation S1  
276 (Section S6, SI). Micropollutant removal efficiency (“measured removal” in Figure 4) was  
277 calculated as difference between inlet and outlet concentrations. Micropollutant removal efficiency  
278 during continuous operation (“predicted removal” in Figure 4) was predicted using removal rate  
279 constant  $k$  ( $\text{d}^{-1}$ ) estimated during batch experiment according to Equation 1:

$$280 \quad \text{Removal (\%)} = \left( 1 - \left( \frac{1}{(1+k_i \cdot \text{HRT})} \right) \right) \cdot 100 \quad \text{Equation 1}$$

281

## 282 **3. Results and discussion**

283

### 284 **3.1 Batch experiment**

285

#### 286 *3.1.1 Denitrification kinetics*

287 Denitrification rates ( $r_{\text{NO}_3,2\text{-N}}$ ,  $k_{\text{NO}_3,2\text{-N}}$ ) were derived through linear regression of measured  $\text{NO}_3^-$ -N  
288 and  $\text{NO}_2^-$ -N concentration during batch experiment (Figure S5, SI). The ethanol-dosed reactor  
289 presented a higher surface-normalized denitrification rate  $r_{\text{NO}_3,2\text{-N}}$  (Table 2) than the methanol driven  
290 one, which is in agreement with previous studies (Santos et al., 2001; Christensson et al., 1994).  
291 This is likely due to the higher growth yield expected using ethanol thereby leading to higher  
292 biomass production per surface area in ethanol-dosed systems (Mokhayeri et al., 2009). On the  
293 other hand, denitrification rates ( $k_{\text{NO}_3,2\text{-N}}$ ) normalized by biomass weight (higher for the ethanol-  
294 dosed reactor) were comparable in the two MBBRs, suggesting similar activity in terms of nitrate  
295 and nitrite removal in the two biofilms.

296

#### 297 *3.1.2 Micropollutant removal kinetics*

298 Biotransformation kinetics of most of the investigated chemicals could be described with first-order  
299 equation (Table 1, process 2), thereby allowing for the estimation of removal rates  $k$  ( $\text{d}^{-1}$ ) and  
300 pseudo-first order biotransformation rate constants  $k_{\text{bio}}$  ( $\text{L g}_{\text{biomass}}^{-1} \text{d}^{-1}$ ). Abiotic transformation  
301 processes were previously investigated by the authors using plastic (polyethylene) carriers  
302 (AnoxKaldnes Z-carriers) and effluent wastewater (Torresi et al., 2016), suggesting no significant  
303 impact of abiotic processes (e.g., abiotic hydrolysis, volatilization, sorption onto plastic or glass) on  
304 the removal of several targeted micropollutants (Figure S9). Figures 1 and S1 summarize measured  
305 and simulated micropollutant concentration profiles during batch experiments.

306 The removals of erythromycin, clarithromycin, venlafaxine, carbamazepine, sulfamethoxazole and  
307 sulfamethizole was predicted using (i) a pseudo-first order biotransformation model (Table 1,  
308 process 2), with no interaction (inhibition/enhancement) between micropollutant and primary  
309 substrate; and (ii) additionally, a cometabolic model (Table 1, process 3), assuming that the  
310 turnover of the micropollutants is enhanced by the presence of primary substrate. Predictions with  
311 the two models are presented in Figure 1b–d using dashed lines and solid lines, respectively. The  
312 goodness of the two model fits ( $R^2$ ) is summarized in Table S4 (SI). For erythromycin and  
313 clarithromycin, the cometabolic model ( $R^2 > 0.9$ ) provided only for a marginal improvement of the  
314 fitting compared to the pseudo-first order biotransformation model ( $R^2 > 0.8$ ), making it difficult to  
315 draw conclusion on the removal mechanism of these two compounds. However, the prediction of  
316 carbamazepine's removal was significantly improved by adopting the cometabolic model ( $R^2 > 0.9$ )  
317 compared to the pseudo first-order biotransformation model ( $R^2 < 0.5$ ) in both MBBRs.  
318 Cometabolic biotransformation of carbamazepine has been previously observed in aerobic and  
319 anoxic activated sludge (Plósz et al., 2012) with cometabolic biotransformation rate constant  $q_{\text{bio}}$   
320 ( $1.2 \text{ L g}^{-1} \text{ d}^{-1}$  under anoxic condition), in close agreement with our results. Similarly, the removal of  
321 venlafaxine, sulfamethoxazole and sulfamethizole removal was better predicted using the  
322 cometabolic model ( $R^2 > 0.9$ ). In Figure 1a, the measured and simulated concentration of soluble  
323 COD (sCOD), nitrate ( $\text{NO}_3\text{-N}$ ) and nitrite ( $\text{NO}_2\text{-N}$ ) and simulated readily biodegradable COD ( $S_S$ )  
324 are reported. For the abovementioned micropollutants,  $S_S$  limitation (approximately after 3 hours  
325 from the beginning of the experiment) corresponded to a change in biotransformation kinetics.  
326 Interestingly, in the ethanol-fed reactor, the modelled  $S_S$  limitation coincided with a significant  
327 decrease in the biotransformation rates of sulfamethoxazole and sulfamethizole (Figure 1d right),  
328 thereby leading to a rather low removal rate during the rest of the experiment. A similar effect for  
329 both compounds, though at lower extent, was observed for the methanol-dosed reactor.

330 Cometabolic transformation of trace chemicals was previously shown in suspended cultures under  
331 aerobic, anaerobic and anoxic conditions (Delgadillo-Mirquez et al., 2011; Fernandez-Fontaina et  
332 al., 2014; Plósz et al., 2010; Popat and Deshusses, 2011; Tran et al., 2013). Removal via  
333 cometabolism was previously observed for sulfamethoxazole in nitrifying (Kassotaki et al., 2016;  
334 Müller et al., 2013) and in aerobic and anoxic activated sludge (Alvarino et al., 2016), as well as for  
335 erythromycin and roxithromycin (Fernandez-Fontaina et al., 2014) in nitrifying activated sludge.  
336 Thus, our results support the hypothesis that the change of primary substrate availability can  
337 significantly impact the removal of a number of micropollutants as a result of cometabolic  
338 mechanisms under denitrifying conditions.

339

340 While the other sulfonamides followed cometabolic biotransformation, the removal of sulfadiazine  
341 was different. It could not be described with first-order kinetics ( $R^2 < 0.2$  and  $< 0.4$  for methanol-  
342 and ethanol-dosed MBBR respectively, dashed lines in Figure 1 e), due to the presence of its  
343 conjugate acetyl-sulfadiazine. Acetyl-sulfadiazine is the main human metabolite of sulfadiazine  
344 (Vree et al., 1995) and it has been previously observed to undergo de-acetylation (Zarfl et al.,  
345 2009), similarly to other acetyl-sulfonamides such as  $N^4$ -acetylsulfamethoxazole (Göbel et al.,  
346 2007). For these chemicals, a model including retransformation (deconjugation) of acetyl-  
347 sulfadiazine to sulfadiazine and concurrent biotransformation of sulfadiazine was used to estimate  
348 biotransformation rate constants  $k_{\text{bio}}$  for sulfadiazine and retransformation rate constant  $k_{\text{Dec}}$  for  
349 acetyl-sulfadiazine (Table 1, processes 1 and 2). However, this modelling approach did not  
350 adequately describe the concentration changes of sulfadiazine in any of the MBBRs ( $R^2 < 0.2$ ,  
351 continuous lines in Figure 1e):

352 For the methanol-dosed MBBR, acetyl- sulfadiazine was decreasing rapidly and sulfadiazine (being  
353 formed from acetyl-sulfadiazine) reached a maximum before being slowly further biotransformed

354 (Figure 1e left). In the ethanol-dosed MBBR, the acetyl-sulfadiazine was also rapidly removed and  
355 sulfadiazine concentration did never increase. Thus, for the ethanol-dosed reactor there is no  
356 indication for a deconjugation reaction of acetyl-sulfadiazine to sulfadiazine, while that is partially  
357 possible in the methanol-dosed one. Hence, the transformation of acetyl-sulfadiazine probably  
358 occurred partially following another metabolic pathway leading to the formation of other  
359 (undetected) transformation products. To test whether other pathways could be possible, the  
360 EAWAG-BBD pathway prediction systems (EAWAG-BBD, 2016) was used: It showed the  
361 possible transformation of acetyl-sulfadiazine to other transformation products, e.g., 2-  
362 aminopyrimidine and 4-aminobenzenesulfonic acid (Figure S6, SI). However, additional research  
363 on the different transformation pathways of acetyl-sulfadiazine in the two tested MBBRs is needed  
364 to substantiate this hypothesis. Further discussion on the biotransformation of acetyl-sulfadiazine  
365 and sulfadiazine in the two investigated MBBRs is reported in S4 in SI.

366

367

368

### **3.2. Influence of dosed carbon-source on microbial communities**

369 We analyzed the biofilm microbial community of the two different MBBRs by 16S rRNA amplicon  
370 sequencing. After implementation of quality control measures, a total of 10847 high quality  
371 sequences were obtained with an average length of 460 bp. Subsequently, the number of reads of  
372 each sample was normalized to 4562 sequences and clustered into an average of 690 observed  
373 OTUs at 97% sequence similarity per sample (cut-off level of 3%). The facultative methanol-  
374 utilizing  $\beta$ -Proteobacteria, *Methylophilus*, was identified as the main relative abundance genus  
375 (24%) in the methanol-dosed reactor (Figure 2)—a result that closely agreed with previous studies  
376 on methanol-dosing denitrification systems (Baytshtok et al., 2009; Jenkins et al., 1987; Lu et al.,  
377 2014). For the biofilm grown in the ethanol-dosed reactor, *Arcobacter* and *Thiothrix* genus  
378 exhibited 23% and 9% relative abundance, respectively. *Arcobacter* was previously identified in

379 full-scale MBBRs treating municipal wastewater (Biswas and Turner, 2012). *Thiothrix* is known to  
380 degrade sulfur containing compounds and it was suggested to influence the removal of sulfa-based  
381 antibiotics in membrane bioreactors (Xia et al., 2012). Microbial community diversity in the two  
382 MBBRs was evaluated by comparing Shannon diversity and evenness indices. We observed no  
383 major difference in the methanol- and ethanol-dosed reactors between Shannon diversity indices  
384 ( $4.153 \pm 0.05$  and  $4.184 \pm 0.03$ , mean and standard deviation, respectively) and evenness ( $0.092 \pm$   
385  $0.002$  and  $0.095 \pm 0.001$ , respectively) (Figure 2, Table S5 of the SI). Similar values of Shannon  
386 diversity indices were found previously in aerobic nitrifying MBBRs (Bassin et al., 2015; Torresi et  
387 al., 2016). On the other hand, the extrapolated taxonomic richness ACE in the methanol- and  
388 ethanol-dosed reactors were estimated to be  $999 \pm 103$  and  $781 \pm 87$  OTUs, respectively, thus  
389 suggesting slightly differences between the microbial richness of the two biofilms (Figure 2).  
390 Similar evidence was obtained from nitrite reductase *nirK*- and *nirS*-based restriction fragment  
391 length polymorphism (RFLP) analysis on activated sludge supplemented with methanol or ethanol,  
392 with higher diversity in terms of richness of *nirS* genotypes observed in the methanol-dosed sludge  
393 (Hallin et al., 2006).

394

### 395 **3.3. Influence of dosed carbon source on micropollutants biotransformation**

396 A comparative assessment of the estimated  $k_{\text{bio}}$ ,  $q_{\text{bio}}$  and  $k_{\text{Dec}}$  values for the methanol- and ethanol-  
397 dosed MBBRs is shown in Figure 3. For 9 compounds the estimated values of biotransformation  
398 and cometabolic transformation rate constants ( $k_{\text{bio}}$  and  $q_{\text{bio}}$ ) for the methanol-dosed MBBR were  
399 higher (1.5 to 2.5-fold) than those from the ethanol-dosed reactor (namely atenolol, citalopram,  
400 trimethoprim, ibuprofen, iopromide, metoprolol, iohexol, iomeprol, sotalol, venlafaxine).  
401 Conversely, the sulfonamides acetyl-sulfadiazine, sulfamethoxazole, sulfamethizole were  
402 transformed at higher rate constants (up to 2.8-fold) in the ethanol-dosed reactor. The remaining 10

403 compounds behaved similarly in both reactors. We further classified the biotransformation potential  
404 of the targeted micropollutants of the two denitrifying MBBRs in three groups (Table 3): easily  
405 degradable ( $q_{\text{bio}}$  and  $k_{\text{bio}} > 2$ ), moderately degradable ( $0.2 \leq k_{\text{bio}}$  and  $q_{\text{bio}} \geq 2$ ) and hardly degradable  
406 ( $k_{\text{bio}}$  and  $q_{\text{bio}} < 0.2$ ).

407 We observed that some of the targeted chemicals classified as easily degradable (propranolol,  
408 atenolol, citalopram) presented removal rate constant  $k$  ( $\text{d}^{-1}$ ) above  $10 \text{ d}^{-1}$  and similar between the  
409 two MBBRs (Table 3). As the two MBBRs presented different amount of biomass (Table 2) during  
410 batch experiment, the lower values of  $k_{\text{bio}}$  for the high degradable compounds in the ethanol-dosed  
411 reactor could mainly derive from the normalization to a higher amount of biomass prevailing in the  
412 ethanol-dosed reactor.

413 On the other hand, a number of moderately degradable chemicals (i.e., X-ray contrast media,  
414 ibuprofen, metoprolol and sotalol) were associated to  $k$  and  $k_{\text{bio}}$  values approximately two times  
415 higher in the methanol-dosed than in the ethanol-dosed reactor. The two biofilm microbial  
416 communities likely played an important role on the biotransformation of these chemicals. As  
417 previously described (Section 3.2), the higher microbial richness observed in the biofilm enriched  
418 with methanol could have likely contributed to the overall higher micropollutant biotransformation  
419 in the methanol-dosed MBBR. Similarly, positive associations between biodiversity and the rates of  
420 specific micropollutant biotransformations were observed in activated sludge (Johnson et al., 2015;  
421 Stadler and Love, 2016) and MBBR (Torresi et al., 2016).

422 No major differences were observed between the biotransformation rate constants  $k_{\text{bio}}$  of the hardly  
423 degradable compounds (diatrizoic acid, iopamidol, diclofenac and phenazone) in the two MBBRs,  
424 suggesting that their removal is neither biomass nor carbon source dependent.

425

### 426 **3.4 Highlighted compounds**

427 Among the X-ray contrast media, iopamidol and diatrizoic acid were found to be recalcitrant in both  
428 reactors during batch experiment (Figure S1, SI), while iomeprol, iohexol and iopromide were  
429 found moderately degradable (Figure 1d). These results are in agreement with previous studies  
430 conducted with aerobic MBBRs (Escolà Casas et al., 2015a; Hapeshi et al., 2013). Our results show  
431 that denitrifying MBBRs could effectively remove iomeprol, iohexol and iopamidol, with  $k_{\text{bio}}$   
432 comparable to studies on activated sludge (Joss et al., 2006; Onesios et al., 2009)

433 The analgesic ibuprofen exhibited lower biotransformation rate constants (Table 3) than the ones  
434 obtained in previous studies under aerobic conditions (Escolà Casas et al., 2015a,b; Falås et al.,  
435 2012; Torresi et al., 2016) as ibuprofen is easily degraded under aerobic conditions. Other  
436 analgesics, i.e., phenazone and diclofenac, have also previously observed to be hardly degradable in  
437 both aerobic MBBR and activated sludge (Escolà Casas et al., 2015a; Joss et al., 2006).  
438 Nevertheless,  $k_{\text{bio}}$  values for diclofenac were reported to be higher under nitrifying conditions in  
439 both biofilms and activate sludge (Torresi et al., 2016; Tran et al., 2009) than as obtained in this  
440 study under denitrifying conditions, thus indicating limited diclofenac removal under anoxic  
441 conditions.

442

443 In the batch experiment, citalopram was fully removed in both reactors within 0.4 days (Figure 1b),  
444 resulting in a  $k_{\text{bio}}$  of  $2.3 \text{ L d}^{-1} \text{ g}_{\text{biomass}}^{-1}$  (Table 3). Similar biotransformation kinetics was found in  
445 aerobic MBBR (Escolà Casas et al., 2015a) and sludge (Suárez et al., 2012), while anoxic CAS  
446 showed lower kientics compared to the one obtained in th anoxic MBBRs of this study. At HRTs  
447 higher than 0.4 days, a removal efficiency of 65% was achieved in a complete autotrophic nitrogen  
448 removal process (Alvarino et al., 2015). In our study, predicted removal efficiency of citalopram (at  
449 HRT of 0.4 days) was calculated to be >80% in both reactors (Equation 1) during continuous-flow

450 operation. Furthermore, in denitrifying activated sludge reactors a  $k_{\text{bio}}$  of  $0.5 \text{ L d}^{-1} \text{ g}_{\text{biomass}}^{-1}$  was  
451 obtained for citalopram (Suárez et al., 2010) and a removal of 44 % under anoxic condition (Suárez  
452 et al., 2010).

453

454 The removal of carbamazepine and venlafaxine (with  $q_{\text{bio}}$  ranging between  $1.1$  and  $1.9 \text{ L g}^{-1} \text{ d}^{-1}$ )  
455 followed the kinetics described by the cometabolic model in our study, as suggested previously (see  
456 Section 3.1.2). With respect to biotransformation kinetics, only one study showed cometabolic  
457 biotransformation rate constants of up to  $2 \text{ L g}^{-1} \text{ d}^{-1}$  for carbamazepine in aerobic and anoxic  
458 activated sludge (Plósz et al., 2012). On the other hand biotransformations rate constants equal to  
459  $0.9 \text{ L g}^{-1} \text{ d}^{-1}$  in aerobic MBBR have been reported (Escolà Casas et al., 2015a). In our study we thus  
460 observed 30 % removal of carbamazepine during batch experiments, which is in agreement with  
461 previous studies on MBBR (Escolà Casas et al., 2015a) and activated sludge (Dawas-Massalha et  
462 al., 2014; Luo et al., 2014; Zupanc et al., 2013). Thus, low removal of carbamazepine in the tested  
463 MBBRs in batch and continuous-flow experiments may be attributed to the limited transformation  
464 in the absence of primary substrates.

465

466 The biotransformation of the targeted sulfonamides was enhanced in the ethanol-dosed MBBR (up  
467 to 1.8-fold higher). As the ethanol-dosed MBBR showed the highest denitrification rates ( $r_{\text{NO}_3,2\text{-N}}$ )  
468 during the batch experiment, the removal of the targeted sulfonamides may be associated with  
469 primary metabolism rather than biofilm composition (i.e., biodiversity). Interestingly, negative  
470 correlation between biotransformation kinetics of sulfonamides and biodiversity was also observed  
471 in nitrifying MBBRs, and their removal was enhanced at higher nitrification rates in thin biofilms  
472 (Torresi et al., 2016). Similarly, the removal of sulfamethoxazole has been previously shown to be  
473 dependent on the primary metabolism under anoxic condition in activated sludge, while negligible

474 effect of primary substrate was observed under nitrifying condition (Alvarino et al., 2016). This  
475 indicates that sulfonamide removal may be influenced by primary metabolism in both nitrifying and  
476 denitrifying conditions.

477

478 Finally, while  $k_{\text{bio}}$  of clarithromycin and erythromycin was found comparable to studies on aerobic  
479 MBBRs (Escolà Casas et al., 2015a), trimethoprim removal occurred with a higher  $k_{\text{bio}}$  under  
480 denitrification conditions than in aerobic MBBRs (Escolà Casas et al., 2015a; Falås et al., 2013).

481

### 482 **3.5 Impact of carbon dosing during continuous-flow operation**

483 A continuous-flow experiment tested different dosing conditions of organic carbon in terms of  
484 primary substrate (methanol or ethanol) and influent loading (variable  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratio)  
485 without adaptation of the biofilm as described in Section 2.6 (details in Section S7, SI). The  
486 removal efficiency of micropollutants did not present any correlation with the tested  $\text{COD}_{\text{added}}/\text{NO}_3\text{-}$   
487  $\text{N}_{\text{influent}}$  ratios and did not significantly differ between the two types of carbon sources (Figure S8,  
488 SI). Notably, only trimethoprim removal increased with increasing carbon availability in the  
489 ethanol-dosed reactor.

490

491 Both MBBR systems exhibited denitrification rates directly proportional to the  $\text{COD}_{\text{added}}/\text{NO}_3\text{-}$   
492  $\text{N}_{\text{influent}}$  ratio (Figure S3, SI). However, at  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratios higher than 4.8 and 3.8 for  
493 the methanol- and the ethanol-dosed MBBR, respectively, denitrification rates did not increase and  
494 similar effluent concentrations of COD were measured for both MBBRs (estimated to be equal to  
495 the inert soluble COD in the influent medium) (Figure S4, SI). This indicates that excess COD  
496 dosing during continuous-flow operation could have been used for internal storage rather than as  
497 primary energy source. This has been previously observed under substrate feast-famine cycles in

498 continuously operated activated sludge (Beun et al., 2000). Similarly, feast-famine conditions  
499 associated to change from high to low  $\text{COD}_{\text{added}}/\text{NO}_3\text{-N}_{\text{influent}}$  ratio during continuous-flow operation  
500 might have influenced the performance of the two post-denitrifying MBBRs in this study.

501

502 Furthermore, the continuous-flow operation experiment was carried out at HRT of 2 h, simulating  
503 HRTs typically operated in denitrification stages in full scale WWTPs, and which might have been  
504 too short to observe differences in the removal of micropollutants. In fact, the batch experiment  
505 showed that the removal of most of the targeted micropollutants (with the exception of the  
506 compounds removed through cometabolism) continued after 2 h from the start of the experiment  
507 (Figure 1), suggesting a possible removal enhancement at higher HRT. Accordingly, the increase of  
508 HRT has been found to enhance the removal of a number of micropollutants in activated sludge  
509 (Maurer et al., 2007; Petrie et al., 2014) and MBBR (Mazioti et al., 2015).

510

### 511 **3.6. Comparison of the batch and the continuous-flow experiment**

512 Figure 4 compares the measured removal efficiencies under continuous –flow operation with the  
513 predicted removal efficiencies. The predicted values were calculated using the removal rates (k)  
514 estimated in the batch experiment according to first-order kinetics (Table 3). As presented in Figure  
515 4, the removal rates (k) estimated from batch experiments allowed predicting of the elimination of  
516 most of the targeted compounds in continuous-flow operation. However, predicted removal  
517 efficiencies did not match the measurements for a number of micropollutants, i.e. sulfamethoxazole,  
518 carbamazepine, atenolol and trimethoprim. A possible explanation for this discrepancy might be  
519 that the removal rates (k) used to predict the removals were obtained by fitting the first order  
520 kinetics, while in reality for some compounds cometabolic or deconjugation approaches are more  
521 appropriate.

522

523 As the biotransformation kinetics of most of the compounds could be described with a first-order  
524 equation (Table 1, process 2 and Figure 1), it could be predicted that an HRT of 2 h (0.083 d) would  
525 only allow a partial removal of the easily biodegradable compounds (e.g., atenolol, trimethoprim  
526 and citalopram) in the continuous-flow experiment (Figure 4). For the compounds following this  
527 type of biotransformation kinetics, it could be predicted (Equation 1, Section 2.6) that the increase  
528 of the HRT up to 6 hours (0.25 d) would improve the removal efficiency by about 20%, achieving  
529 high removals in both reactors (>70%) for all the compounds listed as “easily biodegradable” in  
530 Table 3.

531

#### 532 **4. Conclusions**

533 In order to investigate the removal of micropollutants in denitrifying Moving Bed Biofilm Reactors  
534 (MBBRs), two laboratory-scale MBBRs were tested using nitrified effluent wastewater dosed with  
535 methanol and ethanol, respectively. The following conclusions have been drawn:

536

- 537 • According to the batch experiment, all targeted micropollutants showed biotransformation rate  
538 constants over  $0.2 \text{ L d}^{-1} \text{ g}_{\text{biomass}}^{-1}$  under denitrifying condition, except for diclofenac, phenazone,  
539 diatrizoic acid and iopamidol, which were found to be recalcitrant. Accordingly, it has been  
540 suggested that that HRTs of approximately 6 h could considerably enhance the removal of most  
541 of the targeted micropollutants.
- 542
- 543 • The biotransformation rate constants in the methanol-dosed MBBR were 1.5 to 2.5-fold higher  
544 than in the ethanol-dosed MBBR for 9 out of the 22 spiked pharmaceutical. Oppositely, the  
545 sulfonamides acetyl-sulfadiazine, sulfamethoxazole, sulfamethizole were transformed at higher

546 biotransformation rate constants in the ethanol-dosed MBBR. The rest of the compounds  
547 presented similar biotransformation in both reactors.

548

549 • The removal of venlafaxine, carbamazepine, sulfamethoxazole and sulfamethizole was most  
550 likely enhanced by the presence of organic growth substrates in the beginning of the batch  
551 experiment, suggesting cometabolic removal for these compounds.

552

553 • The continuous-flow experiment conducted at conditions typically operated in full-scale  
554 WWTPs (i.e., HRT =2h) did not show significant correlation between the removal efficiency of  
555 micropollutants and the increase of carbon dosage or type.

556

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563

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565

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762 **Tables**

763 **Table 1. Stoichiometric (Gujer) matrix of the ASM-X (which includes processes such as parent**  
 764 **compound retransformation, biotransformation and the cometabolic model) and two-step denitrifying**  
 765 **model used in this study.**  
 766 **Stoichiometric coefficients: A= (1-Y<sub>H</sub>)/(1.143 \* Y<sub>H</sub>); B = (1-Y<sub>H</sub>)/(1.713 \* Y<sub>H</sub>); F = ratio between molecular**  
 767 **mass of parent compound and metabolite undergoing deconjugation. Parameters and state variables**  
 768 **for determination of micropollutant kinetics are described in the main text. Parameters and state**  
 769 **variables for the denitrifying model are defined in Table S1 in Supplementary Information. For**  
 770 **estimation of denitrification kinetics, biomass concentration X<sub>H</sub> is expressed in gCOD L<sup>-1</sup>; \*Due to**  
 771 **short duration of the batch experiment and low S/X ratio, negligible biomass growth was assumed.**  
 772

(i) Component → i	C <sub>LI</sub>	C <sub>CJ</sub>	S <sub>NO3</sub>	S <sub>NO2</sub>	S <sub>N2</sub>	S <sub>s</sub>	X <sub>biomass</sub>	Process rate
(j)Processes ↓								
<b>Micropollutants kinetics</b>								
<sup>(1)</sup> <b>Parent compound retransformation</b>	F	-1						$\frac{k_{Dec} C_{CJ} X_{biomass}}{1 + K_D X_{biomass}}$
<sup>(2)</sup> <b>Biotransformation</b>	-1							$\frac{k_{bio} C_{LI} X_{biomass}}{1 + K_D X_{biomass}}$
<sup>(3)</sup> <b>Cometabolism</b>	-1							$\frac{q_{bio} (S_s / (S_s + K_S)) + k_{bio} C_{LI} X_{biomass}}{1 + K_D X_{biomass}}$
<b>Denitrification kinetics</b>								
<b>R1</b>			-A	+A	-1/Y <sub>H</sub>		*	$\mu_H \eta_{g1} X_H \frac{S_s}{K_{S1} * S_s} \frac{S_{NO3}}{K_{NO3}^{HB} * S_{NO3}}$
<b>R2</b>				-B	+B	-1/Y <sub>H</sub>	*	$\mu_H \eta_{g2} X_H \frac{S_s}{K_{S2} * S_s} \frac{S_{NO2}}{K_{NO2}^{HB} * S_{NO2}}$
R1: Anoxic growth of heterotrophs, reducing nitrate to nitrite (NO <sub>3</sub> <sup>-</sup> → NO <sub>2</sub> <sup>-</sup> )								
R2: Anoxic growth of heterotrophs, reducing nitrite to nitrogen (NO <sub>2</sub> <sup>-</sup> → N <sub>2</sub> )								

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775 **Table 2. Values of denitrification rates normalized by carriers surface area ( $r_{\text{NO}_{3,2}\text{-N}}$ ) and biomass**  
 776 **concentration ( $k_{\text{NO}_{3,2}\text{-N}}$ ) measured during batch experiments. SA: total surface area of carriers in the**  
 777 **MBBRs.**  
 778

<b>MBBR</b>	<b>SA</b> ( $\text{m}^2$ )	<b>Biomass</b> ( $\text{g L}^{-1}$ )	<b><math>r_{\text{NO}_{3,2}\text{-N}}</math></b> ( $\text{gNO}_{3,2}\text{-N m}^{-2} \text{d}^{-1}$ )	<b><math>k_{\text{NO}_{3,2}\text{-N}}</math></b> ( $\text{gNO}_{3,2}\text{-N g}_{\text{biomass}}^{-1} \text{d}^{-1}$ )
Methanol-dosed	0.2	3.28 $\pm$ 0.93	1.77 $\pm$ 0.92	0.11 $\pm$ 0.06
Ethanol-dosed	0.2	4.20 $\pm$ 0.25	2.32 $\pm$ 0.62	0.11 $\pm$ 0.03

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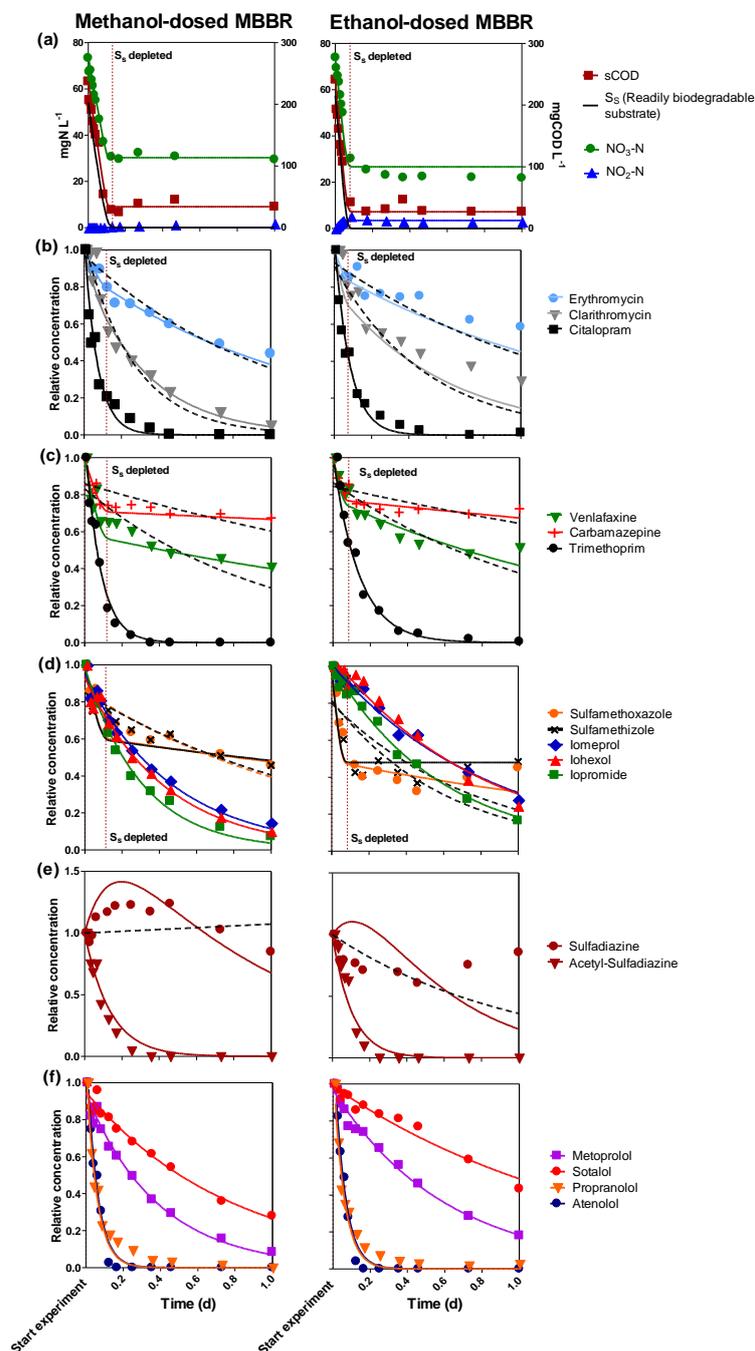
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**Table 3. Values of  $k$ ,  $k_{bio}$ ,  $q_{bio}$  and  $k_{Dec}$  estimated for the two MBBRs from the data obtained in the batch experiments. “ $k$ ” defines the removal rate constant obtained following single first-order kinetics and not accounting for biomass concentration and sorption processes. “ $k_{bio}$ ” and “ $q_{bio}$ ” refer to removal rate constants normalized for biomass and sorption processes. Unindexed values correspond to “ $k_{bio}$ ” (Biotransformation process, Table 1, Process 2). Index (1) indicates the case of retransformation rate constant “ $k_{Dec}$ ” of acetyl-sulfadiazine to sulfadiazine (Transformation process, Table 1, Process 1). Index (2) refers to the cometabolic rate constant “ $q_{bio}$ ” (Cometabolism, Table 1, Process 3). The following abbreviations are used: n.d. = not degradable, SD = standard deviation.**

Compound	Methanol-dosed MBBR		Ethanol-dosed MBBR	
	$k \pm SD$ ( $d^{-1}$ )	$k_{bio}, q_{bio} \pm SD$ ( $L d^{-1} g_{biomass}^{-1}$ )	$k \pm SD$ ( $d^{-1}$ )	$k_{bio}, q_{bio} \pm SD$ ( $L d^{-1} g_{biomass}^{-1}$ )
<b><i>Easily degradable; <math>k_{bio}, q_{bio} &gt; 2</math></i></b>				
Propranolol	17.8 ± 0.2	12.9 ± 1.3	17.7 ± 0.2	11.7 ± 0.7
Atenolol	17.8 ± 0.2	6.4 ± 0.6	17.6 ± 0.2	5.1 ± 0.3
Citalopram	14.2 ± 0.9	4.3 ± 0.5	12.3 ± 0.1	2.3 ± 0.1
Trimethoprim	13.6 ± 0.1	4.1 ± 0.4	9.0 ± 0.1	2.1 ± 0.1
Acetyl-sulfadiazine	12.1 ± 0.3	3.7 ± 0.4 <sup>(1)</sup>	17.6 ± 0.2	4.2 ± 0.3 <sup>(1)</sup>
<b><i>Moderately degradable; <math>0.2 \leq k_{bio}, q_{bio} \leq 2</math></i></b>				
Ibuprofen	4.6 ± 0.1	1.4 ± 0.4	2.3 ± 0.1	0.5 ± 0.03
Clarithromycin	2.9 ± 0.7	1.0 ± 0.2 <sup>(2)</sup> 0.6 ± 0.1	4.4 ± 0.9	0.9 ± 0.2 <sup>(2)</sup> 0.4 ± 0.1
Iopromide	3.0 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	0.4 ± 0.1
Metoprolol	2.6 ± 0.1	0.8 ± 0.2	1.7 ± 0.1	0.4 ± 0.03
Iohexol	2.3 ± 0.1	0.7 ± 0.2	1.2 ± 0.1	0.3 ± 0.1
Iomeprol	2.1 ± 0.1	0.6 ± 0.1	1.2 ± 0.1	0.3 ± 0.1
Sotalol	1.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.2 ± 0.02
Erythromycin	1.5 ± 0.1	0.5 ± 0.1 <sup>(2)</sup> 0.2 ± 0.1	2.5 ± 0.1	0.6 ± 0.1 <sup>(2)</sup> 0.2 ± 0.1
Venlafaxine	6.0 ± 0.1	1.9 ± 0.2 <sup>(2)</sup> 0.1 ± 0.1	4.8 ± 0.1	1.1 ± 0.1 <sup>(2)</sup> 0.1 ± 0.1
Carbamazepine	3.9 ± 0.1	1.2 ± 0.3 <sup>(2)</sup> 0.1 ± 0.1	4.6 ± 0.1	1.1 ± 0.1 <sup>(2)</sup> 0.1 ± 0.1
Sulfamethoxazole	5.6 ± 0.8	1.7 ± 0.2 <sup>(2)</sup> 0.1 ± 0.1	13.5 ± 0.7	3.2 ± 0.2 <sup>(2)</sup> 0.1 ± 0.1
Sulfamethizole	5.8 ± 0.9	1.8 ± 0.2 <sup>(2)</sup> 0.1 ± 0.1	13.8 ± 0.8	3.3 ± 0.2 <sup>(2)</sup> 0
Sulfadiazine	1.9 ± 0.2	0.6 ± 0.1	4.2 ± 0.6	1.0 ± 0.2
<b><i>Hardly or non-degradable; <math>k_{bio}, q_{bio} &lt; 0.2</math></i></b>				
Diatrizoic acid	0.3 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.1
Iopamidol	0.2 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1 ± 0.1
Diclofenac	n.d.	n.d.	n.d.	n.d.
Phenazone	n.d.	n.d.	n.d.	n.d.

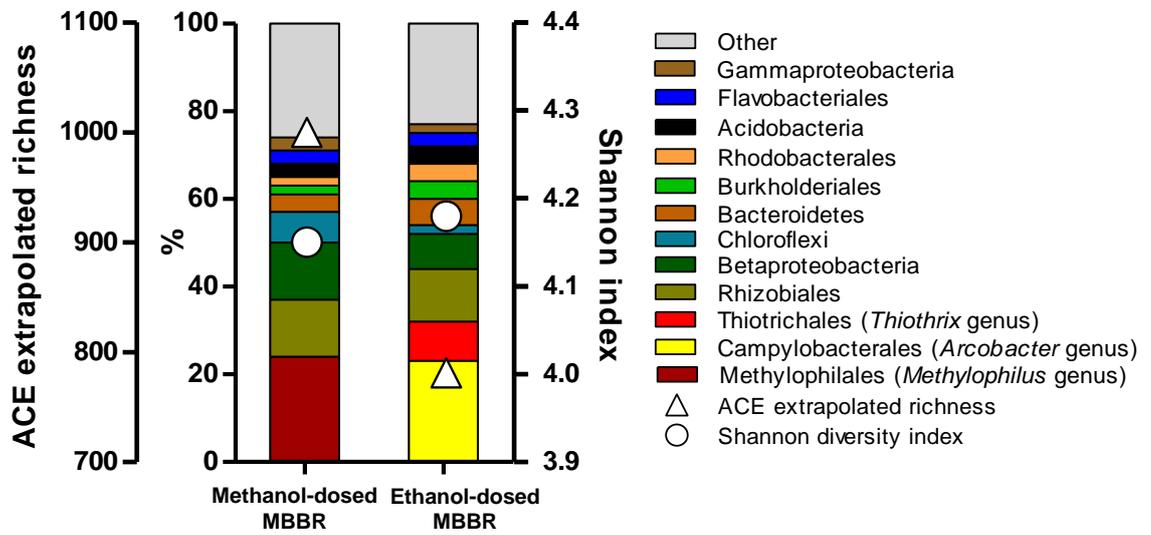
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793 Figure 1. Batch experiment results for selected compounds. On the X-axes “Time (d)” designates the sampling  
 794 time-points. On the Y-axes “Relative concentration” refers to concentrations normalized to the measured  
 795 starting concentrations. Symbols refer to measurements while lines refer to modelling. [a] Macro-pollutants.  
 796 Readily biodegradable substrate ( $S_s$ ) is only modelled. [b-d] Solid lines: modelled concentrations assuming  
 797 cometabolism (process 3, Table 1). Dashed lines: concentrations according to the biotransformation model  
 798 (process 2, Table 1). [e] Solid lines: biotransformation-retransformation model (process 1, Table 1) assuming  
 799 deconjugation of acetyl-sulfadiazine to sulfadiazine. Dashed lines: biotransformation model (process 2,  
 800 Table 1). [f] Solid lines: concentrations according to the biotransformation model (process 2, Table 1).  
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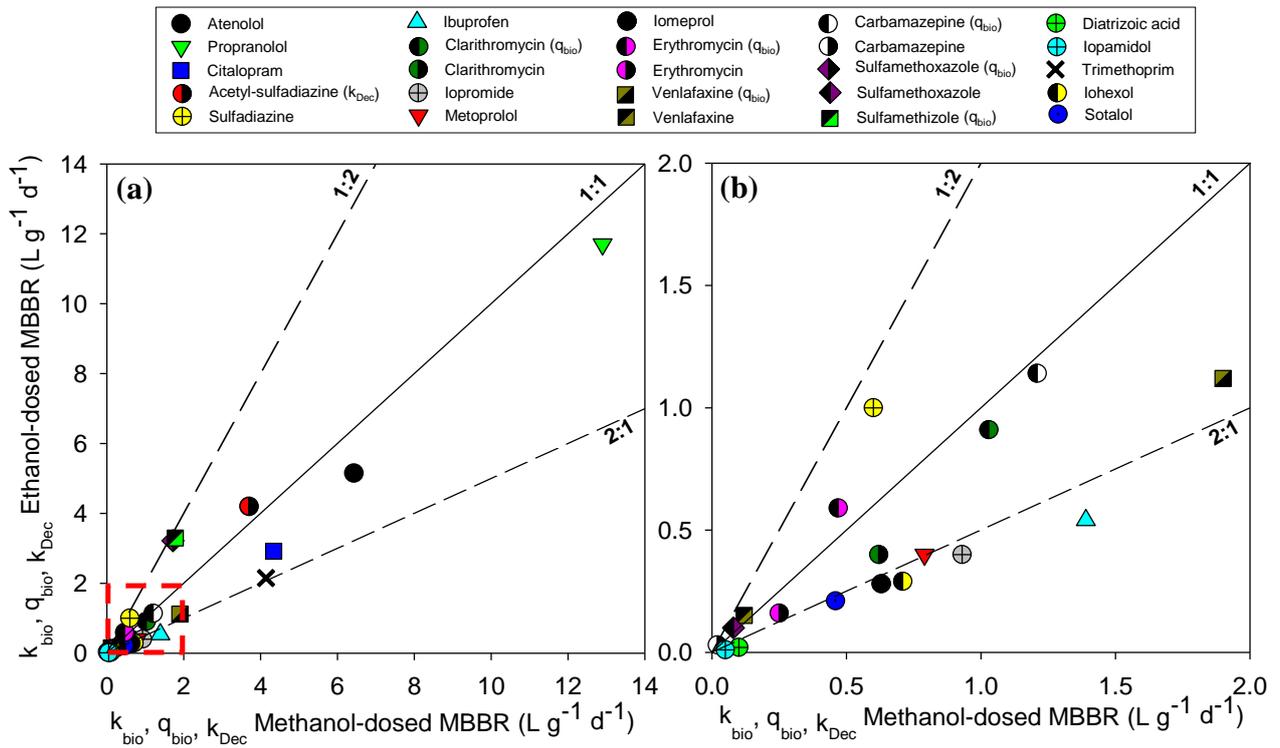
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804 **Figure 2. Order-level taxonomic classification of 16S rRNA amplicons of the two MBBRs. The three**  
805 **most abundant orders are reported also at genus level (*Methylophilus*, *Arcobacter* and *Thiothrix*).**  
806 **Taxa abundance is expressed in percentage (second left axis). Alpha-diversity is measured as ACE**  
807 **extrapolated richness (first left axis) and Shannon diversity index (right axis).**

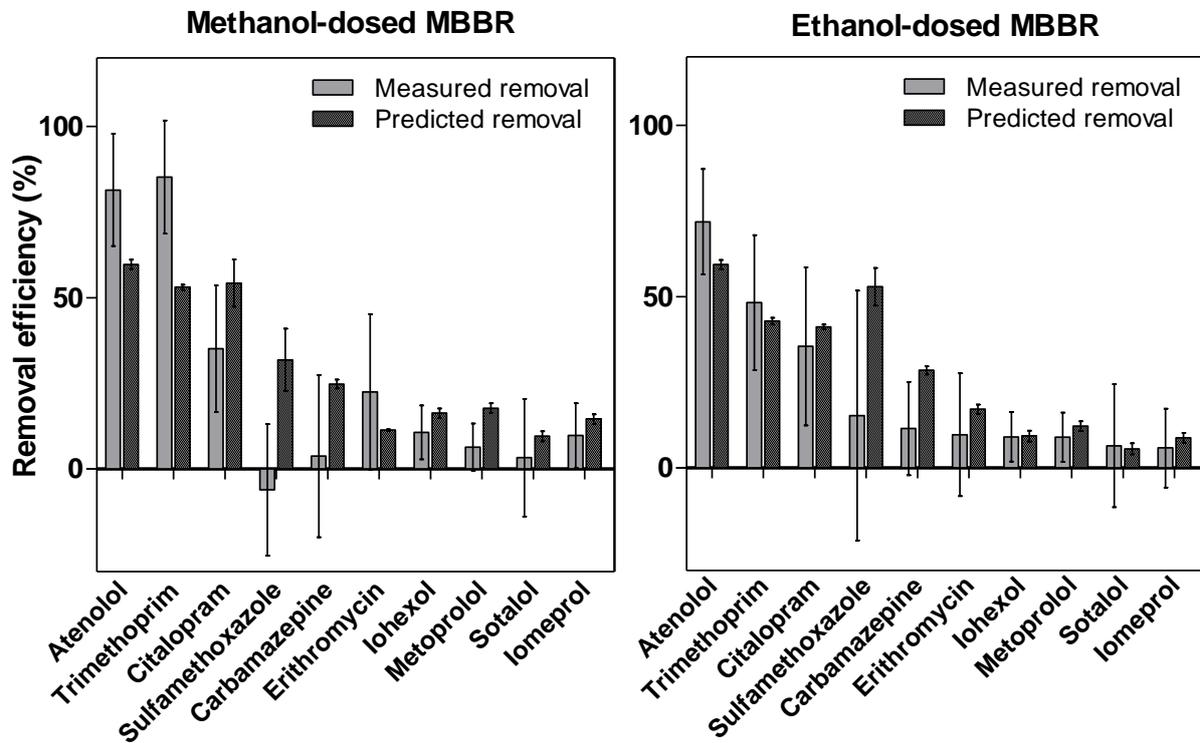
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811 **Figure 3. Comparative assessment between methanol-dosed reactor (x-axes) and the ethanol-dosed**  
 812 **reactor (y-axes) of the removal kinetics  $k_{bio}$ ,  $q_{bio}$  and  $k_{Dec}$  estimated for all targeted compounds (a) and**  
 813 **for compounds with biokinetics ranging between 0 and 2  $L g_{biomass}^{-1} d^{-1}$  (b). Dashed lines (2:1 and 1:2)**  
 814 **delimit area where biokinetics are 2-fold higher or lower than other estimated values. In the legend,**  
 815 **when not specified, symbols refer to estimated  $k_{bio}$ .**

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Figure 4. Measured mean removal efficiency of micropollutants of all the tested  $\text{COD}_{\text{added}}/\text{NO}^3\text{-N}$  influent ratios (presented in Table S3, S1) during the whole continuous-flow experiment, taking into account that no correlation was found between micropollutant removal and  $\text{COD}_{\text{added}}/\text{NO}^3\text{-N}$  influent ratios. The measured removals were calculated as difference between influent and effluent concentration, expressed as a percentage. Predicted removal was based on removal rate constants  $k$  ( $\text{d}^{-1}$ ) derived from batch experiments, calculated according to Equation 1 in Section 2.6.